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IroN, a Novel Outer Membrane Siderophore Receptor Characteristic of *Salmonella enterica*

ANDREAS J. BÄUMLER,[1]* TRACY L. NORRIS,[1] TODD LASCO,[1] WOLFGANG VOIGT,[2] ROLF REISSBRODT,[2] WOLFGANG RABSCH,[3] AND FRED HEFFRON[4]

Department of Medical Microbiology and Immunology, Texas A&M University, College Station, Texas 77843-1114[1]; Robert Koch-Institut[2] and Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin,[3] 38843 Wernigerode, Germany; and Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201-3098[4]

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Speciation in enterobacteria involved horizontal gene transfer. Therefore, analysis of genes acquired by horizontal transfer that are present in one species but not its close relatives is expected to give insights into how new bacterial species were formed. In this study we characterize *iroN*, a gene located downstream of the *iroBC* operon in the *iroA* locus of *Salmonella enterica* serotype Typhi. Like *iroBC*, the *iroN* gene is present in all phylogenetic lineages of *S. enterica* but is absent from closely related species such as *Salmonella bongori* or *Escherichia coli*. Comparison of the deduced amino acid sequence of *iroN* with other proteins suggested that this gene encodes an outer membrane siderophore receptor protein. Mutational analysis in *S. enterica* and expression in *E. coli* identified a 78-kDa outer membrane protein as the *iroN* gene product. When introduced into an *E. coli fepA cir fiu aroB* mutant on a cosmid, *iroN* mediated utilization of structurally related catecholate siderophores, including *N*-(2,3-dihydroxybenzoyl)-L-serine, myxochelin A, benzaldehyde-2,3-dihydroxybenzhydrazone, 2-*N*,6-*N*-bis(2,3-dihydroxybenzoyl)-L-lysine, 2-*N*,6-*N*-bis(2,3-dihydroxybenzoyl)-L-lysine amide, and enterochelin. These results suggest that the *iroA* locus functions in iron acquisition in *S. enterica*.

The genera *Salmonella* and *Escherichia* diverged from a common ancestor some 100 million to 160 million years ago (26). The lineage of the genus *Salmonella* subsequently split into two species, *S. enterica* and *S. bongori* (22, 28). During formation of these species a large amount of DNA was acquired by plasmid- or phage-mediated horizontal gene transfer (3). As a result of horizontal transfer, more than 10% of the *S. enterica* serotype Typhimurium genome consists of genetic material that is not present in *Escherichia coli* (25, 30). To fully comprehend the biological characteristics that distinguish *S. enterica* from closely related species such as *E. coli* and *S. bongori*, it is necessary to study the functions of proteins that are encoded by genetic material that was acquired by way of horizontal transfer.

Genetic material that is absent from closely related bacteria but is present in all phylogenetic lineages of *S. enterica* was likely received during horizontal transfer events that contributed to the formation of this species. Two genetic regions on the *S. enterica* chromosome, *Salmonella* pathogenicity island 2 and the *iroBC* operon, indeed show this phylogenetic distribution (4, 19, 24). In a study on the distribution of *iroB* among 197 bacterial isolates, this gene was found to be present in all *S. enterica* serotypes tested but absent from *S. bongori* serotypes and from 15 other bacterial species tested (4). The phylogenetic distribution thus suggests that the gene products encoded in the *iroA* locus confer properties that set *S. enterica* apart from other bacterial species. What are the characteristics that were obtained by *S. enterica* during acquisition of the *iroA* locus?

* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, Texas A&M University, 407 Reynolds Medical Building, College Station, TX 77843-1114. Phone: (409) 862-7756. Fax: (409) 845-3479. E-mail: abaumler@tamu.edu.

The *iroA* locus was first described in *S. enterica* serotype Typhimurium based on its iron-regulated expression (12). An insertion that created a transcriptional fusion between *lacZ* and the *iroA* locus was mapped close to the *tct* locus (13), an area of the *S. enterica* serotype Typhimurium chromosome that is not present in *E. coli* (31). The first genes of the *iroA* locus, designated *iroBC*, were identified in *S. enterica* serotype Typhi during a genetic screen for genes that are regulated by the iron response regulator Fur (5). Regulation by Fur results in expression of *iroBC* under iron-limited growth conditions. In contrast, during growth under iron sufficiency expression is prevented by binding of the Fur-Fe^{2+} repressor complex to a Fur DNA binding site in the *iroB* promoter region. In addition to *iroBC*, the Fur repressor protein controls expression of some 28 genes in *S. enterica*, including those that function in iron acquisition and some genes involved in defense against oxidative stress (38–40). However, the *iroBC* gene products show homology to proteins that have so far not been associated with iron uptake or defense against oxidative stress in other bacteria. IroB shows homology with bacterial glycosyltransferases, and IroC is a member of the ATP binding cassette (ABC) family of transport proteins (5). Interestingly, IroC has little homology to bacterial ABC transport proteins involved in the import of siderophores but rather shows strong homology to ABC export proteins of the eukaryotic multidrug resistance family. To obtain further clues about the function of genes encoded in the *iroA* locus, we analyzed *iroN*, an open reading frame located downstream of the *iroBC* operon.

MATERIALS AND METHODS

Bacterial strains, media, growth conditions, and outer membrane preparations. A collection of *Salmonella* serotypes representing *S. bongori* and six subspecies of *S. enterica* has been described by Reeves et al. (28). All other bacterial strains used in this study are listed in Table 1. All bacteria were routinely cultured aerobically at 37°C in Luria-Bertani (LB) broth or on LB plates. Anti-

TABLE 1. Bacterial strains used

Strain	Genotype	Reference or source
E. coli K-12		
DH5α	*endA1hsdR17* ($r_K^-m_K^-$) *supE44thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [ϕ*80 dlac* Δ(*lacZM15*)]	15
S17-1 λ*pir*	*prp thi recA hsdR*; chromosomal RP4-2 (Tn*1*::ISR*1 tet*::Mu Km::Tn*7*); λ*pir*	33
AB2847	*aroB malT tsx thi*	17
H5058	*aroB malT tsx thi cir fiu fepA*	K. Hantke
S. enterica serotype Typhimurium		
14028	Wild type (isolated from bovine septicemia)	American Type Culture Collection
CL1509	14028 *aroA*::Tn*10*	8
AR1258	14028 *entB*::Mud*J*	38
IR715	14028 Nal^r	35
AJB20	14028 Nal^r *iroBC*::Km^r	5
AIR49	14028 Nal^r *iroBC*::Km^r *aroA*::Tn*10*	This study
AJB52	14028 Nal^r *iroN*::pGP704	This study
AJB64	14028 Nal^r *iroN*::pGP704 *aroA*::Tn*10*	This study
S. enterica serotype Typhi		
AJB70	Wild type (clinical isolate, San Diego, Calif.)	5
TY21a	*galE viaB rpoS*	14
AJB22	TY21a Nal^r	5
AJB54	TY21a Nal^r *iroN*::pGP704	This study

biotics, when required, were included in the culture medium or plates at the following concentrations: kanamycin, 100 mg/liter; chloramphenicol, 30 mg/liter; and carbenicillin, 100 mg/liter. To create iron-limiting or iron-sufficient growth conditions, 0.2 mM 2,2′-dipyridyl or 0.04 mM $FeSO_4$, respectively, was added. Desferal (desferrioxamine B) was purchased from Ciba Geigy (Basel, Switzerland). *N*-(2,3-Dihydroxybenzoyl)-L-serine (DBS) and benzaldehyde-2,3-dihydroxybenzhydrazone were synthesized and kindly provided by L. Heinisch, Hans-Knölle Institut, Jena, Germany. The myxochelin derivatives 2-*N*,6-*N*-bis(2,3-dihydroxybenzoyl) lysine (9), 2-*N*,6-*N*-bis(2,3-dihydroxybenzoyl) lysine amide (37), myxochelin A (21), myxochelin B, and myxochelin C (36) were synthesized in the L or D configuration and kindly provided by W. Trowitzsch-Kienast and H. D. Ambrosi, Technische Fachhochschule, Berlin, Germany. Cross-feeding with bacterial supernatants and utilization of siderophores was detected by an agar diffusion assay (29). The strain to be tested was poured in 3 ml of 2% Noble agar onto a nutrient broth-dipyridyl (NBD) agar plate. Filter paper disks impregnated with ferrioxamine B (5 μl of a 1-mg/ml solution of Desferal in 0.1 M $FeCl_3$), DBS (5 μl of a 1-mg/ml solution), benzaldehyde-2,3-dihydroxybenzhydrazone (5 μl of a 1-mg/ml solution), or myxochelin derivatives (5 μl of a 1-mg/ml solution) were laid onto the top agar, and after incubation overnight at 37°C, growth stimulation around the filter disk was recorded. Growth promotion in broth culture was determined in NBD supplemented with a 500× siderophore stock solution (1 mg/ml). Bacterial outer membranes were isolated as previously described (17). Outer membrane proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were visualized by Coomassie blue staining.

Recombinant DNA techniques. Plasmid DNA was isolated by using ion-exchange columns from Qiagen. Standard methods were used for restriction endonuclease analyses, ligation, and transformation of plasmid DNA (2). Sequencing was performed with an ALF automated sequencer (Pharmacia).

Suicide vector constructs and isolation of mutants. For mutational analysis of *iroN*, a 0.4-kb *Xba*I-*Kpn*I fragment of pTY961 containing an internal part of the *iroN* open reading frame was introduced into the suicide vector pGP704 (20) to give rise to pTY966. Plasmid pTY966 was conjugated into *S. enterica* serotype Typhimurium IR715 and *S. enterica* serotype Typhi AJB22. Exconjugants were designated AJB52 and AJB54, respectively. Strains AJB64 and AIR49 were generated by P22 transduction of *aroA*::Tn*10* from *S. enterica* serotype Typhimurium CL1509 into strains AJB52 and AJB20, respectively. *E. coli* S17-1 λ*pir* was used for propagation of all suicide vector constructs and as a donor for introduction of these constructs into IR715 or AJB22 by conjugation. Chromosomal DNA of mutants was routinely tested by Southern hybridization with suitable DNA probes to confirm mutational inactivation of the gene of interest.

Southern hybridization. The inserts of plasmids pTY961 and pTY911 (5) were used to generate DNA probes specific for *iroN* and *iroCDE*, respectively. Chromosomal DNA was isolated as recently described (2). Chromosomal DNA of strains shown in Fig. 4 was restricted with *Eco*RI, and the fragments were separated on a 0.5% agarose gel. Southern transfer of DNA onto a nylon membrane was performed as previously described (2). Hybridization was performed at 65°C in solutions without formamide. Two 15-min washes were performed under nonstringent conditions at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS. Hybrids were detected by using a labeling and detection kit (nonradioactive) from Boehringer Mannheim.

Computer analysis. The nucleotide sequences were compared to SWISS-PROT, PIR, and GenPept at the National Center for Biotechnology Information by using the program blastX and to GenBank and EMBL by using the program blastN (1). Multiple alignments were performed with the program CLUSTAL, which is part of the program package PCGENE.

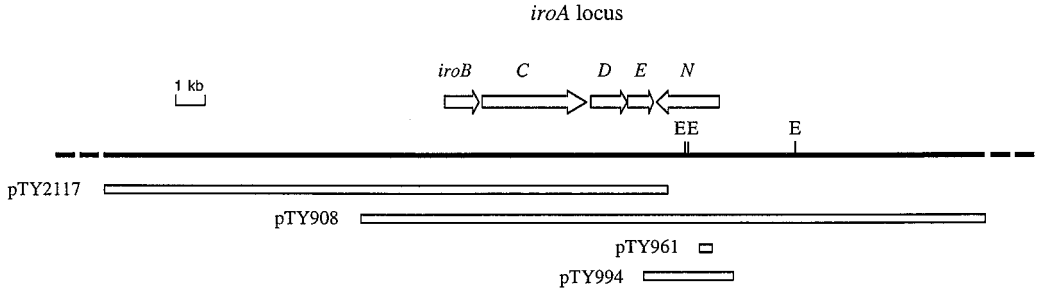

FIG. 1. Restriction map of a DNA region located at about four centisomes on the *S. enterica* serotype Typhi chromosome. Positions and sizes of inserts carried in cosmids (pTY908 and pTY2117) or plasmids depicted were determined previously (5). Arrows above the map indicate positions and orientations of open reading frames identified by sequence analysis. E, *Eco*RI.

	IroN	PfeA	BfeA	FepA	Cir
IroN	100				
PfeA	52	100			
BfeA	52	53	100		
FepA	52	59	49	100	
Cir	35	36	19	35	100

FIG. 2. Percentage sequence identity determined by pairwise alignment of amino acid sequences from IroN, PfeA, BfeA, FepA, and Cir with the program CLUSTAL.

Nucleotide sequence accession number. The sequence reported (Fig. 1) has been deposited at GenBank under accession no. U97227.

RESULTS

Sequence analysis of a DNA region located downstream of *iroBC*. The nucleotide sequence of a 4,837-bp DNA region of *S. enterica* serotype Typhi AJB70 that is located downstream of *iroBC* was determined. Directly downstream of *iroC* were two open reading frames transcribed in the same orientation. These open reading frames were designated *iroDE* (Fig. 1). The close proximity of the open reading frames and the lack of transcriptional terminators suggest that the *iroBCDE* cluster forms an operon. The deduced amino acid sequence of *iroD* showed 28% sequence identity with Fes, the *E. coli* enterochelin esterase (27). The amino-terminal 169 amino acids of IroE displayed 38% identity with the deduced amino acid sequence of an open reading frame located downstream of *pfeA*, the enterochelin receptor gene of *Pseudomonas aeruginosa* (data not shown) (10). Downstream of *iroDE* was a third open reading frame transcribed in the opposite orientation. This open reading frame, termed *iroN*, encoded a polypeptide of 727 amino acids with a calculated molecular mass of 79.5 kDa. Cleavage of a predicted N-terminal signal sequence of 25 amino acids would yield a mature protein with a calculated molecular mass of 76.8 kDa. The region between *iroE* and *iroN* contained a putative transcriptional terminator (stem bp 2261 to 2270; loop bp 2271 to 2273; stem bp 2274 to 2283 of the GenBank sequence). A putative Fur-DNA binding site (4576-GATAATTATTATCATTAGC-4558) that matches the *E. coli* consensus sequence (34) in 16 of 19 bases was located 86 bp upstream of the *iroN* start codon. The G+C content of the entire 10,837-bp DNA region containing the *iroBCDE* and *iroN* genes was 55%, which is slightly higher than the 52% average G+C content characteristic of *S. enterica.*

Sequence homology identified IroN as a member of the family of TonB-dependent outer membrane receptor proteins. The highest degree of sequence identity was found with outer membrane receptor proteins that mediate uptake of the siderophore enterochelin (Fig. 2). Multiple alignment between IroN and enterochelin receptors from *E. coli* (FepA) (23), *Bordetalla pertussis* (BfeA) (6), and *P. aeruginosa* (PfeA) (10)

```
BfeA    MSTP--RFALHYASASVLLAASGLAMAQTATQIHDPSQVQQMATVQVLGT
PfeA    MSSRA----LPAVPFLLLSSCLLANAVHAAGQGDGSVIELGEQTVVA--T
FepA    MNKK-------IHSLALLVNLG--IYGVAQAQEPTDTPVSHDDTIVV--T
IroN    MGMRVKKFIWL-----ITVVSTGINSPLSAAESTD----DNGETIVVEST
        *                 .         .         . *. .  *

BfeA    AEEEIKESLGVSVITAEEIARRPPTNDLSDLIRREPGVNLTGNSASGARG
PfeA    AQEETKQAPGVSIITAEDIAKRPPSNDLSQIIRTMPGVNLTGNSSSGQRG
FepA    AAEQNLQAPGVSTITADEIRKNPVARDVSKIIRTMPGVNLTGNSTSGQRG
IroN    AEQVLKQQPGVSIITRDDIQKNPPVNDLADIIRKMPGVNLTSNSASGTRG
        * .   .  *** ** ..* . *   *.. .**  ******.**.** **

BfeA    NSRQVDIRGMGPENTLILIDGKPVTSRNAVRYGWNGDRDTRGDTNWVPAE
PfeA    NNRQIDIRGMGPENTLILVDGKPVSSRNSVRYGWRGERDSRGDTNWVPAD
FepA    NNRQIDIRGMGPENTLILIDGKPVSSRNSVRQGWRGERDTRGDTSWVPPE
IroN    NNRQIDIRGMGPENTLVLIDGVPVTSRNSVRYSWRGERDTRGDTNWVPPE
        *.**.***********.*.** **.***.** .* *.**.****.***..

BfeA    EVERIEVIRGPAAARYGSGAMGGVVNIITKRPADRATGSITYYTNQPEDS
PfeA    QVERIEVIRGPAAARYGNGAAGGVVNIITKQAGAETHGNLSVYSNFPQHK
FepA    MIERIEVLRGPARARYGNGAAGGVVNIITKKGSGEWHGSWDAYFNAPEHK
IroN    MVERIEMIRGPAAARYGSGAAGGVVNIITKRPTNDWHGSLSLYTNYPESS
         .****..**** ****.** *********.      *.   * * *.

BfeA    REGNTNRVNARISAPIS-DTLS-MRLYGNYNKTNPDARDINAGHANTSDN
PfeA    AEGASERMSFGLNGPLTENL--SYRVYGNIAKTDSDDWDINAGHESNRTG
FepA    EEGATKRTNFSLTGPLGDEF--SFRLYGNLDKTQADAWDINQGHQSARAG
IroN    KEGDTRRGNFSLSGPLAGDTLSTMRLYGNLNRTDADSWDINSS------A
         ** . * .  . .*.  .      *.***  .*..* .*** .

BfeA    GNPST--AGREGVINQDLSALFSWKADSHNTVDLDMGFSRQGNLFAGDTM
PfeA    KQAGTLPAGREGVRNKDIDGLLSWRLTPEQTLEFEAGFSRQGNIYTGDTQ
FepA    TYATTLPAGREGVINKDINGVVRWDFAPLQSLELEAGYSRQGNLYAGDTQ
IroN    GTKNA--AGREGVTNKDINSVFSWKMTPQQILDFEAGYSRQGNIYAGDTQ
           .  ****** *.*. ..  *   . . .... *.*****...***

BfeA    NNANSDFSDSLY--GKETNAMYRENYALTHRGVYDWGTSRAS-VGYDYTR
PfeA    NTNSNNYVKQML--GHETNRMYRETYSVTHRGEWDFGSSLA-YLQYEKTR
FepA    NTNSDSYTRSKY--GDETNRLYRQNYALTWNGGWDNGVTTSNWVQYEHTR
IroN    NSNSNAVTKSLAQSGRETNRLYRQNYGLTHNGIWGWGQSRLG-FYYEKTD
        *  .          * *** .**. *..*  *  . * .      *. *

BfeA    NARQREGLAGGPEGA--PTAG-GYDTARLKNWRAAAEASVPFHLGFEQVA
PfeA    NSRINEGLAGGTEGIFDPNNA-GFYTATLRDLTAHGEVNLPLHLGYEQTL
FepA    NSRIPEGLAGGTEGKFNEKATQDFVDIDLDDVMLHSEVNLPIDFLVNQTL
IroN    NTRMNEGLSGGGEGR--ITNDQTFTTNRLTSYRTSGEVNVPVIWLFEQTL
        *.*  ***.** **          .    *      .* ..*     .*

BfeA    TVGVEWLRESLEDPAGTRQTYTGGAIGGTA---PADRDPKSRQTSYALFA
PfeA    TLGSEWTEQKLDDPSSNTQ--NTEEGGSIPGLAGKNRSSSSSARIFSLFA
FepA    TLGTEWNQQRMKDLSSNQALTGTNTGGAIDGVSTTDRSPYSKAEIFSLFA
IroN    TVGAEWNRDELNDPSSTSLTVKDSNIAGIPG-SAANRSSKNKSEISALYV
        *.* **  . . * ..          ..      .* . .     .*.

BfeA    EDNIEIDERTMLTPGVRLDHNSEFGSNWSPSLNASYAVTD--ALKGGIAR
PfeA    EDNIELMPGTMLTPGLRWDHHDIVGDNWSPSLNLSHALTERVTLKAGIAR
FepA    ENNMELTDSTIVTPGLRFDHHSIVGNNWSPALNISQGLGDDFTLKMGIAR
IroN    EDNIEPMAGTNIIPGLRFDYLSESGSNFSPSLNLSQELGEFVKVKAGIAR
        *.*.*    * . **.* *     * * **.** *  . .   .* ****

BfeA    AYKAPNLYQSNPNYLLYSRGNGCLASQTNTNGCYLVGNEDLSPETSVNKE
PfeA    AYKAPNLYQLNPDYLLYSRGQGCYGQ---STSCYLRGNDGLKAETSVNKE
FepA    AYKAPSLYQTNPNYILYSKGQGCYAS---AGGCYLQGNDDLKAETSINKE
IroN    AFKAPNLYQTSEGYLLYSKGNGC-PKDITSGGCYLVGNKNLDPEISINKE
        *.***.***  .  *.***.*.**      . .*** **  * .* *.***

BfeA    IGFEYDPGTWRTSMAYFRNDYRNKIVAGTDVQYRLANG------ARVLQW
PfeA    LGIEYSHDGLVAGLTYFRNDYKNKIESGLSPVDHASGGKGDYANAAIYQW
FepA    IGLEFKRDGWLAGVTWFRNDYRNKIEAGYVAVGQNAVGTD------LYQW
IroN    IGLEFTVDDYHASVTYFRNDYQNKIVAGDQIIGRSASG------AYVLQW
        .*.*.  .   .... *****.*** .*     . .  *       . **

BfeA    TNSGKAVVEGLEGNLFIPLASN-LDWNTNFTYMIQSKEKATGEPLSVIPE
PfeA    ENVPKAVVEGLEGTLTLPL-ADGLKWSNNLTYMLQSKNKETGDVLSVTPR
FepA    DNVPKAVVEGLEGSLNVPV-SETVMWTNNITYMLKSENKTTGDRLSIIPE
IroN    QNGGKALIEGIEASMAVPLMPDRLNWNTNATYMITSEQKDTGNPLSIIPK
         *  **..**.*. . .*. .. . *  * ***. * .* **. **. *

BfeA    YTINSTLDWFYTPQLSFQANLTYYGKQEGPSTNVRTGVELNGDGRQTISP
PfeA    YTLNSMLDWQATDDLSLQATVTWYGKQKPKKYDYH-GDRVTGSANDQLSP
FepA    YTLNSTLSWQAREDLSMQTTFTWYGKQQPKKYNYK-GQPAVGPETKEISP
IroN    YTVNTFLDWTITNALSANVNWTLYGKQKPRTHAESRSEETKGLSGKALGA
        **.*. * *     ** .   * ****          .    *     ...

BfeA    YALAGLSMGYEVNRNLKFRVGVSNLFDKQLYREGNA------------SS
PfeA    YAIAGLGGTYRLSKNLSLGAGVDNLFDKRLFRAGNAQGVVG------IDG
FepA    YSIVGLSATWDVTKNVSLTGGVDNLFDKRLWRAGNAQTTGDLAGANYIAG
IroN    YSLVGANVNYDINKNLRLNVGISNIFDKQIYR----------------SA
        *.. *      . .*. .  *. *.***.. *                 .

BfeA    AGAATYNEPGRAYYATATVSF
PfeA    AGAATYNEPGRTFYTSLTASF
FepA    AGAYTYNEPGRTWYMSVNTHF
IroN    EGANTYNEPGRAYYAGVTASF
         ** *******. *      *
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FIG. 3. Multiple sequence alignment of IroN, PfeA, FepA, and BfeA with the program CLUSTAL. Dashes represent gaps introduced by the program to improve the alignment; identical amino acids are indicated by asterisks; dots indicate amino acids with similar properties.

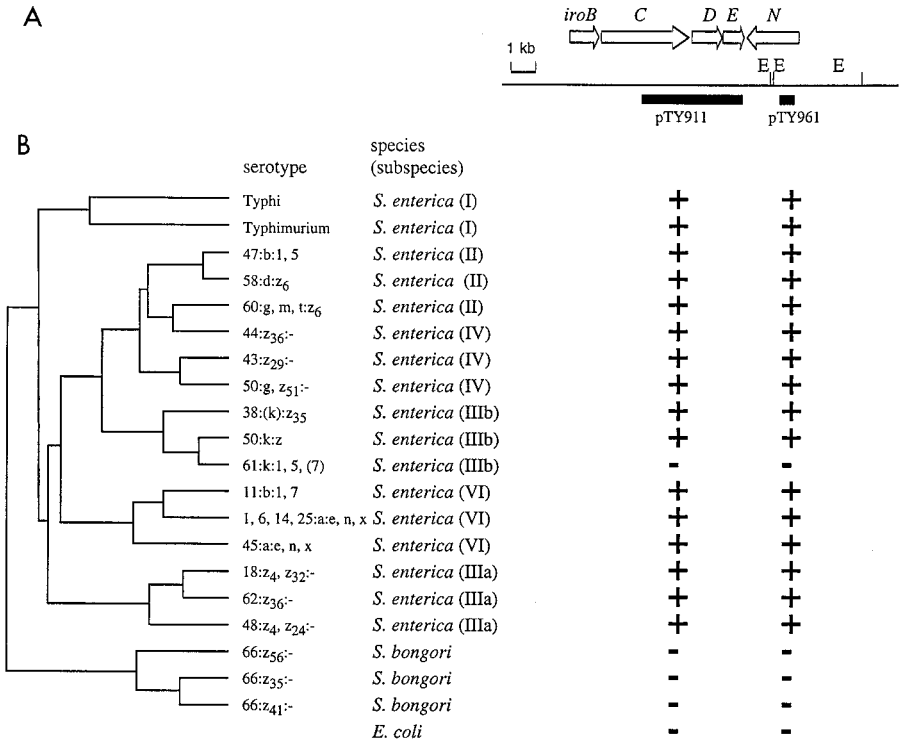

FIG. 4. Phylogenetic distribution of genes of the *iroA* locus. The phylogenetic tree on the left was established by Reeves and coworkers (28). (A) Restriction map of the region from *S. enterica* serotype Typhi AJB70 (E, *Eco*RI). Positions of genes (arrows) identified in the *iroA* locus and of DNA fragments used as probes (black bars) are indicated. (B) Results of hybridization with these DNA probes. +, hybridization signal; −, no hybridization signal.

showed that 36% of the amino acids were identical in all four receptors (Fig. 3). The conservation of amino acid sequences was strongest between amino acids 77 and 171 of IroN, where all four receptors had 85% identical amino acids.

Phylogenetic distribution of *iroN*. To obtain information on the distribution of *iroN*, we used 20 strains representing all phylogenetic lineages of the genus *Salmonella*, including *S. bongori* and *S. enterica* subspecies I, II, IIIa, IIIb, IV, and VI. The phylogenetic relationship among these strains has previously been established by multilocus enzyme electrophoresis (28). Analysis of the distribution of *iroN* among these strains can therefore identify the branch of the phylogenetic tree in which this gene was acquired. To compare the distribution of *iroN* with that of other genes of the *iroA* locus DNA, probes specific for *iroCDE* (pTY911), and *iroN* (pTY961) were used for Southern hybridization (Fig. 4).

The distribution of *iroB* among 197 bacterial isolates collected in Germany revealed the presence of this gene in all *S. enterica* isolates tested. However, *iroB* was absent from 26 bacterial isolates representing 16 different species, including the closely related organisms *S. bongori* and *E. coli* (4). Like *iroB*, the genes *iroCDE* and *iroN* were present in all lineages of *S. enterica* but absent from *S. bongori* and *E. coli*, as shown by Southern blot analysis with probes pTY911 and pTY961, respectively (Fig. 4). Only one strain of *S. enterica* subspecies IIIb did not contain the genes *iroCDE* and *iroN*. The phylogenetic distribution of the *iroA* locus is most likely the result of acquisition of *iroBCDE* and *iroN* by a single horizontal transfer event in a lineage ancestral to *S. enterica*. Subsequent loss of the *iroA* locus by deletion is infrequent and was detected only in *S. enterica* subspecies IIIb serotype 61:k:1,5,(7). A possible mechanism for acquisition by way of horizontal transfer is suggested by the presence of a phage attachment site (*atdA*) located close to *iroA* in *S. enterica* (32). However, alternate scenarios that could explain the phylogenetic distribution of *iroA* (e.g. deletion of the *iroA* locus from *S. bongori* and *E. coli*) cannot at this point be ruled out.

Identification of the *iroN* gene product. Homologies to siderophore receptors from other bacteria suggested that the *iroN* gene product is localized in the outer membrane. To detect IroN in outer membrane preparations, we constructed mutants of *S. enterica* serotype Typhimurium (AJB52 and AJB64) and *S. enterica* serotype Typhi (AJB54) in which the *iroN* open reading frame was disrupted by integration of suicide vector pTY966 via homologous recombination. *S. enterica* serotype Typhi and *S. enterica* serotype Typhimurium have been shown to contain three major iron-regulated outer membrane proteins which are 69, 78, and 83 kDa in size, respectively (7, 11). The 69- and 83-kDa proteins likely represent the *S. enterica* FhuA and FepA receptor proteins, respectively. Inactivation of *iroN* in AJB52, AJB54 (data not shown), and AJB64 (Fig. 5) resulted in loss of the 78-kDa outer membrane protein. These data therefore identify the 78-kDa outer membrane protein as the *iroN* gene product. The size predicted for the mature IroN protein (76.8) is in good agreement with the apparent molecular weight determined by SDS-PAGE. Furthermore, a 78-kDa protein could be detected in outer membrane preparations of strain H5058 upon introduction of a cosmid (pTY908) carrying the *iroN* gene of *S. enterica* serotype Typhi (Fig. 5), indicating that IroN also localizes to the outer membrane when expressed in *E. coli*. Outer membrane preparations of *E. coli* strains that were lacking the *iroN* gene [H5058 or H5058 (pTY2117)] did not contain this 78-kDa protein. Sequence analysis of the insert of cosmid pTY908 revealed no open

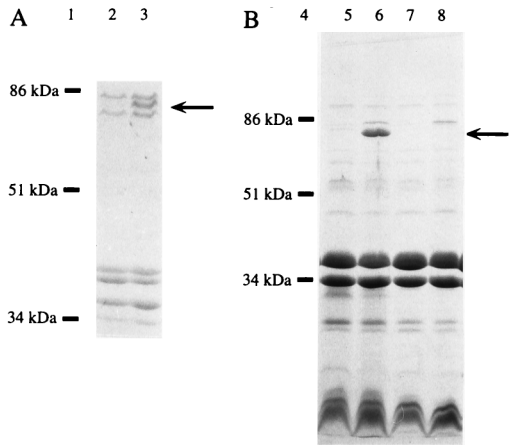

FIG. 5. Outer membrane profiles of bacterial strains which carry the *iroN* gene (lanes 3, 5, and 6) or in which *iroN* is lacking (lanes 7 and 8) or inactivated (lane 2). The position of IroN is indicated by an arrow. Positions and sizes of bands from standard proteins are indicated (lanes 1 and 4). (A) SDS-PAGE of outer membrane preparations of *S. enterica* serotype Typhimurium AJB64 (lane 2) and IR715 (lane 3) grown under iron limitation. (B) Outer membrane profiles of *E. coli* H5058 (lanes 7 and 8) and H5058(pTY908) (lanes 5 and 6) grown in LB supplemented with 0.2 mM 2,2′-dipyridyl (lanes 6 and 8) or 0.04 mM $FeSO_4$ (lanes 5 and 7).

reading frames other than *iroN* that could encode this 78-kDa outer membrane protein (3a).

The presence of a Fur DNA binding site in the *iroN* promoter region suggested that expression of this gene is iron regulated. To investigate iron responsiveness of IroN expression, outer membrane profiles of strain H5058(pTY908) were compared after growth in LB supplemented with either 0.04 mM $FeSO_4$ (iron sufficiency) or 0.2 mM 2,2′-dipyridyl (iron deficiency). This analysis revealed that expression of IroN is repressed in iron-rich medium and strongly induced during growth under iron deficiency (Fig. 5). Thus, IroN is a typical iron-regulated outer membrane protein, as shown by its molecular weight, sequence homology, and iron-regulated expression.

IroN serves as a receptor for catecholate siderophores. The effect of mutations in *iroN* on siderophore utilization was tested in *S. enterica* serotype Typhimurium strains carrying a mutation in *aroA*. *S. enterica aroA* mutants are unable to produce the siderophore enterochelin and therefore exhibit strongly reduced growth under iron deficiency (NBD plates). Levels of growth stimulation of strains CL1509 (*aroA*) and AJB64 (*aroA iroN*) and AIR49 (*aroA iroBC*) by different siderophores were compared on NBD agar plates (Table 2). In *E. coli*, the catecholate-type siderophore enterochelin, composed of a circular trimer of DBS, is transported across the outer membrane via the receptor protein FepA. During its transport into the cytosol, enterochelin is hydrolyzed by Fes esterase to *N*,*N*′,*N*″-tri-(2,3,-dihydroxybenzoyl)-dipeptide (DBS_3), dimers (DBS_2), and monomers of DBS. These breakdown products of enterochelin can be used as siderophores, and each is translocated across the outer membrane by any of three different *E. coli* outer membrane receptor proteins: FepA, Cir, and Fiu (16). Since IroN showed the highest degree of homology to enterochelin receptors of *E. coli*, *B. pertussis*, and *P. aeruginosa*, we investigated the ability of an *S. enterica* serotype Typhimurium *iroN* mutant to utilize catecholate-type siderophores, including enterochelin, DBS, benzaldehyde-2,3-dihydroxybenzhydrazone, 2-*N*,6-*N*-bis(2,3-dihydroxybenzoyl)-L-lysine, 2-*N*,6-*N*-bis(2,3-dihydroxybenzoyl)-L-lysine amide, myxochelin A, myxochelin B, and myxochelin C (Fig. 6). As a control, we studied uptake of ferrioxamine B, a hydroxamate siderophore. All siderophores stimulated growth of all *S. enterica* strains, although the growth stimulation by benzaldehyde-2,3-dihydroxybenzhydrazone and DBS was less in the *iroN aroA* mutant (AJB64) than in the *aroA* mutant (CL1509) and the *iroBC* mutant (AIR49) (Table 2). When utilization of myxochelin A was tested in broth culture, addition of the siderophore promoted growth of an *S. enterica* serotype Typhimurium *iroBC* mutant (AIR49) better than of an *iroN* mutant (AJB64) (Fig. 7). These data indicated that IroN contributes to but is not the sole receptor involved in myxochelin A uptake in *S. enterica.*

To study siderophore transport via IroN in the absence of other *S. enterica* outer membrane receptors that are likely to have overlapping substrate specificities (e.g., FepA or Cir) (18, 38), we used *E. coli* H5058. Due to a mutation in *aroB*, H5058 is unable to produce the siderophore enterochelin. In addition, H5058 carries mutations in the genes *fepA*, *cir*, and *fiu* and is therefore deficient for enterochelin and DBS uptake. The *iroN* gene was introduced into *E. coli* via either a cosmid (pTY908) or a 2,691-bp *Sal*I-*Pst*I fragment cloned into vector pBluescript SK (pTY994) (Fig. 1). Expression of the cloned *iroN* gene of *S. enterica* serotype Typhi (pTY908 and pTY994) in *E. coli* H5058 conferred the ability to utilize several catecholate siderophores during growth under iron deficiency (NBD), including DBS, enterochelin, benzaldehyde-2,3-dihydroxybenzhydrazone, 2-*N*,6-*N*-bis(2,3-dihydroxybenzoyl)-L-lysine, 2-*N*,6-*N*-bis(2,3-dihydroxybenzoyl)-L-lysine amide, and myxochelin A (Table 2). Growth of H5058(pTY994) but not of H5058 was promoted by

TABLE 2. Utilization of siderophores by *S. enterica* and *E. coli* strains

Substance	Growth zone around filter disk placed on NBD agar (mm)						
	S. enterica			*E. coli*			
	CL1509	AJB64	AIR49	H5058(pTY2117)	H5058(pTY908)	AB2847	H5058(pTY994)
Ferrioxamine B	30	30	30	4	4	4	4
Benzaldehyde-2,3-dihydroxybenzhydrazone	16	8	16	0	22	ND	14
Enterochelin	28	28	28	0	15	ND	16
DBS	22	12	22	0	21	15	21
2-*N*,6-*N*-Bis(2,3-dihydroxybenzoyl)-L-lysine	40	40	40	0	22	22	22
2-*N*,6-*N*-Bis(2,3-dihydroxybenzoyl)-L-lysine amide	38	38	ND[a]	0	14	24	15
Myxochelin A	38	38	ND	0	10	22	12
Myxochelin B	30	28	ND	0	0	21	0
Myxochelin C	14	18	ND	0	0	10	0

[a] ND, not determined

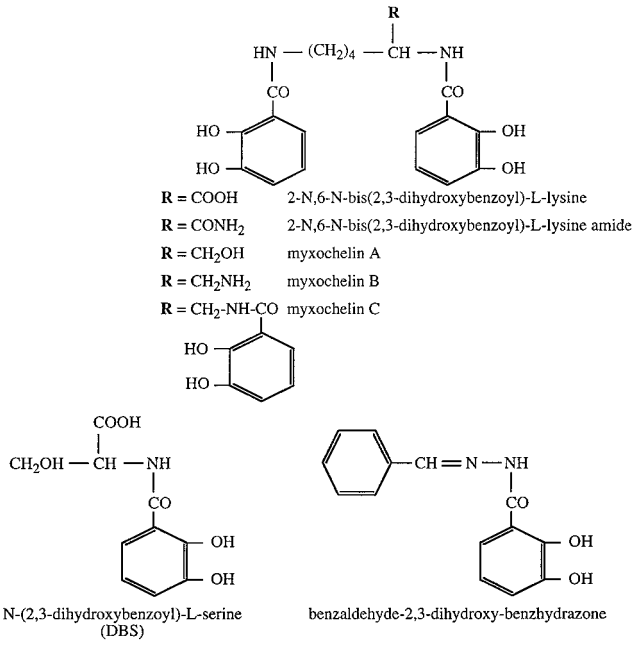

FIG. 6. Structures of catecholate siderophores used in this study.

myxochelin A in broth culture (Fig. 7). These data show that IroN can serve as an outer membrane siderophore receptor which can in part complement mutations in the *E. coli fepA*, *cir*, and *fiu* receptor genes.

All siderophores transported by *E. coli* H5058(pTY908) and H5058(pTY994) possessed a N-linked (2,3-dihydroxybenzoyl) moiety, suggesting that substrate specificity of IroN is restricted to substances containing this group. No differences were observed between the utilization of D and L configurations of myxochelin derivatives (data not shown). However, myxochelin B and C, two siderophores that are closely related to myxochelin A, were not transported by IroN, an indication that additional structural features are required for the interaction between IroN and its substrate. In this context, it should be mentioned that for myxochelin derivatives, substrate specificity was strongly influenced by which group was linked to the C-1 atom of lysine. For instance, presence of a carboxy, hydroxy (myxochelin A), or amide group on C-1 allowed utilization of the respective siderophore via IroN, whereas derivatives substituted at C-1 by an amino (myxochelin B) or a *N*-(2,3-dihydroxybenzoyl) moiety (myxochelin C) were not utilized (Fig. 6).

DISCUSSION

Several lines of evidence suggest that the genes *iroBCDE* and *iroN*, part of the *S. enterica iroA* locus, form a functional unit. Expression of *iroBCDE* and that of *iroN* are both iron regulated, indicating that these genes may be functionally linked. The iron response regulator Fur is likely involved in iron-responsive expression of genes in the *iroA* locus, as typical Fur DNA binding sites are present in the *iroB* and *iroN* promoter regions (5). Both the *iroBCDE* operon and the *iroN* gene are present in *S. enterica* but are absent from closely related bacteria (4) (Fig. 4). This phylogenetic distribution can best be explained by acquisition of the entire *iroA* locus during a single horizontal transfer event in a lineage ancestral to the species *S. enterica*. A function of the *iroA* locus in iron acquisition is suggested by homologies of *iroDE* and *iroN* to genes associated with siderophore utilization. The *iroN* gene encodes an outer membrane siderophore receptor with high homology to FepA, BfeA, and PfeA, the enterochelin receptors of *E. coli*, *B. pertussis*, and *P. aeruginosa*, respectively (Fig. 2 and 3) (6, 10, 23). The *iroD* gene product shows homology to Fes, an *E. coli* enzyme involved in enterochelin utilization (27). Finally, *iroE* has homology to an open reading frame located downstream of *pfeA*, the enterochelin receptor gene of *P. aeruginosa* (10). Although these data suggest that the *iroA* locus functions in iron acquisition, there is at present no evidence implicating the *iroBC* genes in siderophore biosynthesis or uptake.

Acquisition of the *iroN* gene by *S. enterica* introduced two characteristics which now set this species apart from related bacteria. First, localization of IroN in the outer membrane exposes this protein to the host immune system. Fur-regulated siderophore receptors of *S. enterica* serotype Typhimurium are highly expressed during infection, as shown by in vivo studies

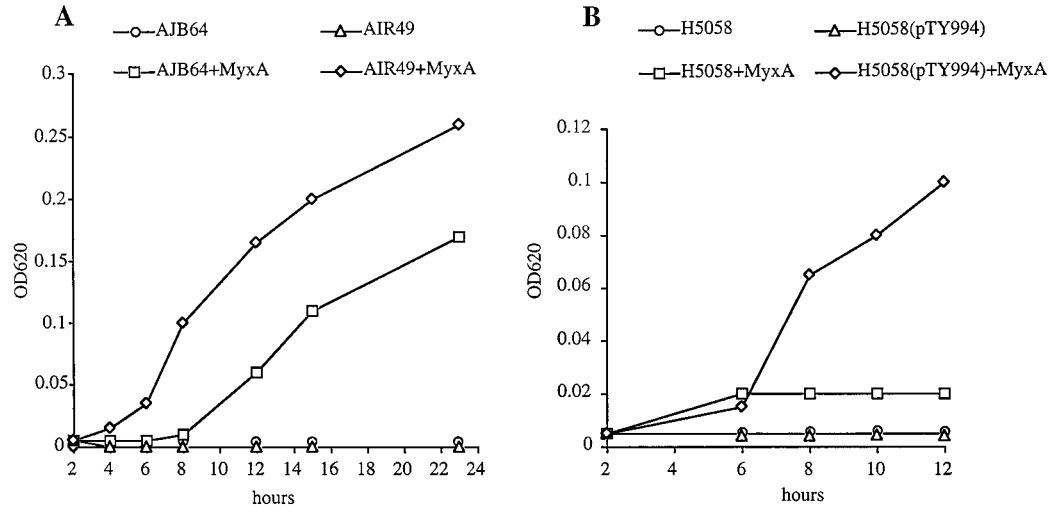

FIG. 7. Growth curves of *S. enterica* serotype Typhimurium (A) and *E. coli* (B) strains in NBD broth culture without supplements or supplemented with myxochelin A (MyxA). Growth was measured as optical density at 620 nm (OD620).

(18). Furthermore, antibodies against iron-regulated outer membrane proteins have been shown to be present in sera from patients who recovered from typhoid fever, an infection caused by *S. enterica* serotype Typhi (11). Thus, acquisition of *iroN* introduced a new antigen that is characteristic of *S. enterica* and likely presents a target for the host immune system. Second, acquisition of *iroN* provided *S. enterica* with a new protein involved in iron uptake. Some of the substrates that are utilized by the *S. enterica* IroN receptor protein are excreted by soil bacteria. For instance, the siderophores 2-*N*,6-*N*-bis(2,3-dihydroxybenzoyl)-L-lysine and 2-*N*,6-*N*-bis(2,3-dihydroxybenzoyl)-L-lysine amide are produced by *Azotobacter vinelandii* (9), and myxochelin A is a product of the myxobacterium *Angiococcus disciformans* (21). It could therefore be speculated that IroN facilitates growth of *S. enterica* in soil, a step frequently encountered during the fecal oral transmission of this ubiquitous pathogen. Although it is possible that some soil bacteria produce siderophores which are transported exclusively by IroN in *S. enterica*, this was not the case for the catecholates used in this study (Table 2). However, the presence of IroN resulted in an increased growth rate of *S. enterica* in broth culture containing myxochelin A (Fig. 7), suggesting that this receptor may confer a selective advantage in the environment under certain growth conditions.

In *E. coli*, IroN mediated uptake of a variety of catecholate siderophores, including DBS, enterochelin, benzaldehyde-2,3-dihydroxybenzhydrazone, 2-*N*,6-*N*-bis(2,3-dihydroxybenzoyl)-L-lysine, 2-*N*,6-*N*-bis(2,3-dihydroxybenzoyl)-L-lysine amide, and myxochelin A. However, transport of these siderophores was not abolished in a *S. enterica iroN* mutant. A possible explanation for the utilization of catecholate siderophores by an *S. enterica iroN* mutant is that the substrate specificity of IroN overlaps with that of other siderophore receptor proteins present in serotype Typhimurium, such as orthologs of FepA and Cir (18, 38). Overlapping substrate specificities of catecholate receptors were first identified in *E. coli*, where DBS is transported by the outer membrane receptors FepA, Cir, and Fiu (16). Furthermore, all catecholates transported by *E. coli fepA cir fiu* mutants expressing IroN [H5058(pTY908) and H5058(pTY994)] were also utilized by the isogenic *E. coli* parent (AB2847), indicating that these siderophores are substrates of the *E. coli* FepA, Cir, and/or Fiu receptor proteins (Table 2). In analogy, uptake of catecholate siderophores in an *S. enterica iroN* mutant may thus be mediated by the orthologs of the FepA and/or Cir receptor proteins present in this organism (18, 38).

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